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Physiological Functioning of Carbonic Anhydrase in the Hydrothermal Vent Tubeworm *Riftia Pachyptila*

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Abstract. On the basis of our experiments, it is clear that carbonic anhydrase (CA) plays an important role in the CO_2-concentrating mechanisms in *Riftia pachyptila*. Plume tissue from freshly collected animals had the highest CA activity, 253.7 ± 36.0 μmol CO_2 min^{-1} g^{-1} wet wt, and trophosome activity averaged 109.4 ± 17.9 μmol CO_2 min^{-1} g^{-1} wet wt. Exposure of living worms to ethoxyzolamide, a carbonic anhydrase inhibitor, resulted in a 99% decrease in CA activity (from 103.9 ± 38.6 to 0.7 ± 0.2 μmol CO_2 min^{-1} g^{-1} wet wt in the plume tissue and 57.6 ± 17.9 to 0.04 ± 0.11 μmol CO_2 min^{-1} g^{-1} wet wt in the trophosome) and essentially a complete cessation of ΣCO_2 uptake. High concentrations of CA appear to facilitate the equilibration between inorganic carbon (C_i) in the external and internal environments, greatly enhancing the diffusion of CO_2 into the animal. In summary, *R. pachyptila* demonstrates very effective acquisition of inorganic carbon from the environment, thereby providing the symbionts with large amounts of CO_2. This effective acquisition is made possible by three factors: extremely effective pH regulation, a large external pool of CO_2, and, described in this paper, high levels of carbonic anhydrase.

Introduction

The giant hydrothermal vent tubeworm *Riftia pachyptila* has an unusual mode of existence, relying primarily upon its symbionts for nutrition. These symbiotic bacteria have been shown to fix only CO_2, yet they have a low affinity for it (K_m for CO_2 = 21–35 μmol l^{-1}; Scott *et al.*, 1994). *R. pachyptila* must, therefore, supply inorganic carbon to the bacteria, which are far removed from the external medium, at high rates and at concentrations sufficient to support its extremely high growth rates (up to 3 ft y^{-1}; Lutz *et al.*, 1994). Net rates of carbon fixation in *R. pachyptila* can be quite high, and the acquisition of inorganic carbon (C_i) *via* CO_2 diffusion has been documented (Childress *et al.*, 1991; Goffredi *et al.*, 1997). Although the P_{CO_2} in the vent environment is higher than in most seawater environments, these animals appear to use carbon-concentrating mechanisms for C_i uptake. Although the worms use binding proteins (hemoglobins) to concentrate oxygen and sulfide internally, there is no evidence that proteins in the extracellular fluids bind either CO_2 or HCO_3^- (Arp *et al.*, 1985, 1987; Childress *et al.*, 1991, 1993; Kochevar *et al.*, 1993; Toulmond *et al.*, 1994). Thus, other concentrating mechanisms must be responsible for the internal accumulation of inorganic carbon.

Many organisms have evolved carbon-concentrating mechanisms that create gradients favoring the internal movement of CO_2, either by increasing the external CO_2 pool or by decreasing the internal CO_2 pool, to aid in the acquisition of CO_2 (Raven, 1991). Carbonic anhydrase (CA) can function in this capacity and has proven to be very important for marine autotrophs. CA, the enzyme that catalyzes the interconversion between carbon dioxide (CO_2) and bicarbonate (HCO_3^-), is widespread among organisms. Since its discovery in the 1930s, CA has been shown to participate in a variety of physiological and biochemical processes, although it is still most commonly known for aiding in CO_2 elimination in metazoans (Meldrum and Roughton, 1933; Maren, 1967; Burnett, 1984; Burnett and McMahon, 1985; Henry 1987, 1988). In addition to CO_2 elimination, however, CA plays an important role in the

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uptake of C_i in aquatic photosynthetic organisms, including a variety of marine plants, algae, eubacteria, and cyanobacteria, as well as in invertebrate hosts of photosynthetic symbionts, including corals, anemones, and clams (Maren, 1967; Lucas and Berry, 1985; Badger, 1987; Weis *et al.*, 1989; Weis, 1991).

Recently, high levels of CA activity have also been measured in the gas-exchange and symbiont-containing tissues of vesicomyid clams, mytilid mussels, and vestimentiferan tube worms from sulfide-rich environments, including hydrothermal vents and cold seeps (Kochevar and Childress, 1996). It has been suggested that CA in these hosts of chemoautotrophic symbionts is important for the uptake of inorganic carbon, as it is for hosts of photosynthetic symbionts (Kochevar, 1992; Kochevar *et al.*, 1993; Kochevar and Childress, 1996). Of all the hosts of chemosynthetic symbionts measured in previous studies, the vent tubeworm, *Riftia pachyptila*, has demonstrated the highest CA activity, especially in the plume (the primary gas-exchange tissue), and the trophosome tissue (the specialized organ in which the bacteria are housed in the trunk of the worm) (Kochevar *et al.*, 1993; Kochevar and Childress, 1996).

The purpose of this project was to determine the physiological functioning of CA in the *R. pachyptila* symbiosis. Based on the assumption that CA in the worm's plume is involved in concentrating C_i from the external environment into the animal, our goal was to provide evidence of the actual *in vivo* functioning of CA in the uptake process. We further assumed that CA enhances CO_2 movement into the worm and that inhibition of CA activity would reduce the rate of CO_2 diffusion, thus lowering the CO_2 uptake rate and internal CO_2 level in *R. pachyptila*. To test these hypotheses, we analyzed the uptake of CO_2 by these animals in the presence and absence of the membrane-permeable carbonic anhydrase inhibitor ethoxyzolamine (EZ). We chose EZ because it has a high *in vivo* stability as well as a high affinity for CA (Holder and Hayes, 1965; Maren, 1967, 1977).

Materials and Methods

Collections

Individuals of *R. pachyptila* were collected at an average depth of 2600 m by submersible during research expeditions to 9°N (9°50′N, 104°18′W) and 13°N (12°48′N, 103°57′W) along the East Pacific Rise in 1994 and 1996. Animals were brought to the surface in a temperature-insulated container and transferred to cold seawater (5°C) in a refrigerated van on board ship. Worms were then sorted to be used either for experiments on living animals (described below) or for immediate measurements of physiological parameters. Live-animal experiments were initiated within 2 h of surfacing. Worms labeled "freshly collected" in this text were dissected in less than 4 h after collection from the sea floor (see Goffredi *et al.*, 1997, for dissection techniques). Tissue and blood samples were stored at −70°C for assays of CA activity.

Analytical methods

ΣCO_2 and ΣH_2S of body fluids and large-vessel water samples were measured in 0.5-ml aliquots with a Hewlett Packard 5880A gas chromatograph (GC) (Childress *et al.*, 1984; "Σ" is used to indicate total concentrations, including all ionic species present). Body fluid pH values were measured with a thermostatted (10°C) Radiometer BMS-2 blood pH analyzer equipped with a G299A capillary pH electrode. All values are given as mean ± standard error.

Carbonic anhydrase activity was measured using the protocol described in Weis *et al.* (1989). Briefly, tissue samples were homogenized in 10 × vol of chilled 25 mmol l^{-1} veronal buffer (25 mmol l^{-1} barbital buffer, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} dithiothreitol, and 10 mmol l^{-1} $MgSO_4$, pH 8) and centrifuged for 10 min at 1200 × *g*. Homogenates were stored on ice and 1-ml aliquots were transferred to test tubes for assay. This homogenate was combined with 1 ml 50 mmol l^{-1} veronal buffer solution and 1 ml CO_2-saturated distilled water. The rate of pH change following the addition of CO_2-saturated distilled water was monitored over time. As a control to determine nonspecific changes in pH, the assay was performed on boiled homogenate. The difference between the boiled and non-boiled sample was considered CA activity (and was measured in triplicate). CA activity was measured as Δ pH min^{-1} $weight^{-1}$; however, to facilitate comparison between the rates of CO_2 conversion and the rates of CO_2 uptake, we converted these units to μmol CO_2 min^{-1} $weight^{-1}$ by assuming 0.351 Δ pH min^{-1} $weight^{-1}$ ≈ 1 μmol CO_2 converted to $HCO_3^- + H^+$ min^{-1} $weight^{-1}$, a factor previously determined by Kochevar and Childress (1996). Protein concentration in crude homogenates was determined spectrophotometrically by the Lowry method, with bovine serum albumin as the standard (Lowry *et al.*, 1951).

Internal vs. external parameter experiments

Experiments in which we were concerned with comparing internal versus external parameters, such as pH and ΣCO_2, were carried out in vessels with a capacity of 6 l to accommodate large worms (see Goffredi *et al.*, 1997 for further details). Use of large (14–42 cm in length and 10–80 g) worms was necessary to measure internal parameters in both vascular and coelomic fluids, as well as in tissues. Seawater was taken from two reservoir tanks, 200 l and 75 l, and pumped by high-pressure, diaphragm metering pumps through the vessels at flow rates ranging from 4 to 12 l h^{-1}, at pressures around 21.5 MPa. All worms in these experiments were first maintained in aquaria with flowing

surface seawater (ΣCO_2 = 2.1 mmol l^{-1}, P_{CO_2} = 0.04 kPa, pH = 8.2, at 8°C). Low internal levels of ΣCO_2, which resulted from maintenance in surface seawater, were necessary to measure the uptake rates of CO_2 over time. Decrease in extracellular ΣCO_2 was monitored over periods of 2–4 days, after which the animals were transferred to experimental vessels.

Experimental water conditions were similar to *in situ* vent conditions of 3.98 ± 0.12 mmol l^{-1} ΣCO_2, 0.15 ± 0.02 mmol l^{-1} ΣH_2S, 0.26 ± 0.03 mmol l^{-1} O_2, 0.38 ± 0.02 mmol l^{-1} N_2, and pH 6.0 ± 0.1. Water ΣCO_2, O_2, and N_2 were controlled by bubbling gases directly into a gas equilibration column, which fed the high-pressure pumps, which supplied the vessels (see diagram in Kochevar *et al.*, 1992; Goffredi *et al.*, 1997). Sulfide concentrations were controlled by continuously pumping anaerobic solutions of sodium sulfide (30–50 mmol l^{-1}) into the gas equilibration column. To stabilize and control the pH, 10 mmol l^{-1} MOPS (3-[*N*-morpholino]propanesulfonic acid) or MES (2-[*N*-morpholino]ethanesulfonic acid) buffer was added to the sulfide solutions. Pressure gauges and sample ports were connected to the vessel outflow to allow monitoring of pressure and water conditions.

Control worms (n = 5) were those animals kept in the above conditions without exposure to inhibitors. Inhibitor-exposed worms (n = 5) were those animals initially maintained in the same manner as for the control worms, and transferred to the same experimental conditions; however, once inside the experimental vessels they were exposed to ethoxyzolamide, a specific inhibitor of carbonic anhydrase (final concentration of 0.8–0.9 mmol l^{-1} in the surrounding water; Holder and Hayes, 1965; Maren, 1967, 1977). EZ was first dissolved in pH 13 seawater (at 4–5 mmol l^{-1} concentrations) and introduced into the vessels over a 1-h time period *via* metering pumps. The flow rate determined the final concentration. Worms were sacrificed at times between 0 and 6 h for the determination of tissue CA and vascular blood and coelomic fluid pH and ΣCO_2.

Flux experiments

For experiments in which we measured metabolite fluxes, we used a smaller flow-through, high-pressure respirometry system (Kochevar *et al.*, 1992). Individuals of *R. pachyptila*, ranging in size from 2 to 10 g, were maintained in a high-pressure vessel (volume = 350 ml). A second aquarium, which did not contain worms, was used as a control. In a gas equilibration column, seawater was bubbled with gas mixes of CO_2, H_2S, O_2, and N_2, which were controlled by a gas flow controller (Sierra Instruments Inc.; Kochevar *et al.*, 1992). Dissolved gas concentrations of 3.11 ± 0.32 mmol l^{-1} ΣCO_2, 0.37 ± 0.03 mmol l^{-1} ΣH_2S, 0.40 ± 0.03 mmol l^{-1} O_2, and 0.35 ± 0.02 mmol l^{-1} N_2 were achieved. A seawater pH of 6.25 ± 0.13 was maintained by a proportional pH controller (Cole-Parmer, Inc.). From the equilibration column, seawater was pumped into the vessels *via* high-pressure pumps, at 12.5 ± 1.0 ml min^{-1}, and maintained at 27.5 MPa *via* gas-charged back pressure valves (Circle Seal Controls, Inc.). A pair of pneumatically actuated 4-port valves (VALCO, Inc.) were used to direct the effluent streams (one at a time) to a gas extraction system, where the effluent was acidified and stripped of dissolved gases. Dissolved gas concentrations were measured using a residual gas analyzer (Hiden Analytical), and pH was measured using a double-junction pH electrode. The minimum flux of CO_2, H_2S, and O_2 discernible by this technique is 0.9 μmol g^{-1} h^{-1}, and 0.4 μmol g^{-1} h^{-1}, respectively. The difference in C_i, oxygen, and sulfide concentrations between the experimental and control vessels were used to calculate the flux rates.

To test the effects of EZ on C_i flux, the worms were first maintained under the conditions described above until they showed signs of autotrophy (stable net C_i uptake), after about 20 h. In this study, we use the word *autotrophy* strictly with respect to carbon. EZ was then added at a concentration of 2 mmol l^{-1} to the surrounding seawater. At the end of the experiment, which lasted approximately 4 days, the worms were weighed and dissected. Tissues were kept in liquid nitrogen for later determination of CA activity.

Results

Freshly collected animals

Plume tissue from freshly collected animals had a CA activity of 253.7 ± 36.0 μmol CO_2 min^{-1} g^{-1} wet wt; trophosome activity averaged 109.4 ± 17.9 μmol CO_2 min^{-1} g^{-1} wet wt. (Table 1). Both body wall and vestimentum had much lower values, which is in agreement with previous research (Table 1; Kochevar and Childress, 1996). Protein values for plume and trophosome tissue averaged 87.7 ± 6.3 mg g^{-1} (n = 27) and 124.7 ± 9.9 mg g^{-1} (n = 16), respectively. Thus, protein-specific CA activities for plume and trophosome were measured to be 4.0 ± 0.9 μmol

Table 1

Carbonic anhydrase activities in Riftia pachyptila

Sample	n	CA activity (μmol CO_2 min^{-1} g^{-1} wet wt)
Plume	38	253.7 ± 36.0
Trophosome	39	109.4 ± 17.9
Body wall	17	5.4 ± 0.6
Vestimentum	6	2.3 ± 0.7

Carbonic anhydrase activity (mean ± SE) for *Riftia pachyptila* collected at 9° and 13°N along the East Pacific Rise. CA activity was measured using the protocol described in Weis *et al.* (1989).

CO_2 min^{-1} mg protein $^{-1}$ (n = 27) and 0.8 ± 0.2 μmol CO_2 min^{-1} mg protein^{-1} (n = 16), respectively.

Internal vs. external parameter experiments

All experimental worms were first kept in maintenance vessels with surface seawater conditions, and monitored until extracellular fluid ΣCO_2 was reduced to a low value (<10 mmol l^{-1}; about 2–4 days). When control worms were subsequently exposed to flowing water with an average ΣCO_2 of 3.98 ± 0.12 mmol l^{-1} and P_{CO_2} of 5.8 ± 0.6 kPa (typical vent conditions), they demonstrated an increase in extracellular fluid ΣCO_2 (Fig. 1a, b). The rate of increase in internal ΣCO_2, from 0 to 6 h, was 4.1 mmol $l^{-1}h^{-1}$ for coelomic fluid and 4.8 mmol $l^{-1}h^{-1}$ for vascular blood. Despite this increase in internal ΣCO_2, there was no change in the pH of either coelomic fluid or vascular blood; values averaged 7.21 ± 0.07 and 7.04 ± 0.07, respectively, during the 6-h time period (Fig. 1c, d).

Worms exposed to EZ were kept in the same overall water conditions as the control worms, with an additional 1 h exposure to 0.8–0.9 mmol l^{-1} EZ. These worms, when placed in typical vent conditions (*i.e.*, high ΣCO_2 and P_{CO_2}), did not accumulate internal ΣCO_2 as rapidly as the control worms (0.8 mmol l^{-1} h^{-1} and 0.0 mmol l^{-1} h^{-1} for coelomic fluid and vascular blood, respectively; Fig. 1a, b). The decreased rate of ΣCO_2 accumulation for vascular blood in the EZ-exposed worms was significantly less than that of the control worms (P = 0.032, ANCOVA; Fig. 1b). EZ-exposed worms, like the control worms, showed no change in the pH of either coelomic fluid or vascular blood during the experiment (7.15 ± 0.09 and 7.07 ± 0.01, respectively), and neither was significantly different from that of the control worms (P = 0.47 and P = 0.72, respectively, Mann-Whitney U test; Fig. 1c, d).

EZ exposure also resulted in a decrease in CA activity of about 99%: from 103.9 ± 38.6 to 0.7 ± 0.2 μmol CO_2 min^{-1} g^{-1} wet wt in the plume tissue and 57.6 ± 17.9 to 0.04 ± 0.11 μmol CO_2 min^{-1} g^{-1} wet wt in the trophosome (Table II).

Flux experiments

For convention, since we are referring to materials that are consumed from the surrounding water, any flux that occurs from the environment into the worm is expressed as a negative value, and any flux that occurs from the worm into the environment is expressed as a positive value. Ini-

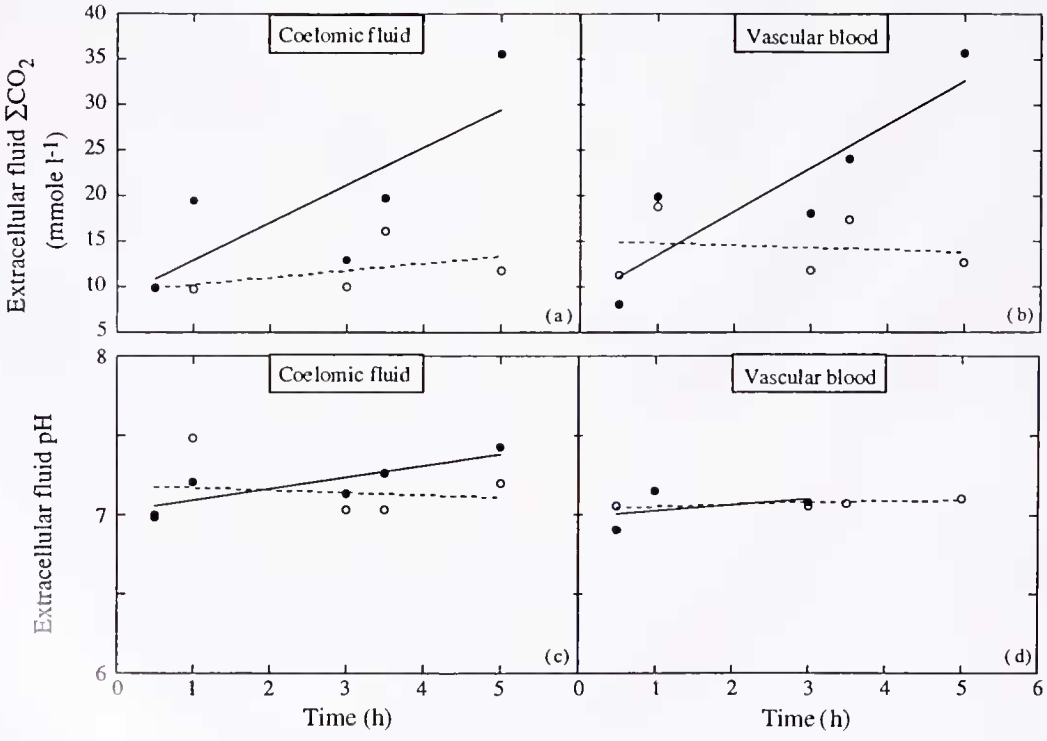

Figure 1 Coelomic fluid ΣCO_2 (a) and pH (c), and vascular blood ΣCO_2 (b) and pH (d), over time for *Riftia pachyptila* exposed to 3.98 ± 0.12 mmol l^{-1} ΣCO_2, 0.15 ± 0.02 mmol l^{-1} ΣH_2S, 0.26 ± 0.03 mmol l^{-1} O_2, 0.38 ± 0.02 mmol l^{-1} N_2, and a pH of 6.0 ± 0.1. Each point represents a single animal sacrificed at the indicated time. Closed symbols represent control worms (n = 5) and open symbols represent worms exposed to ethoxyzolamide (n = 5). Lines are drawn to show correlations (the only significant regression correlations (P < 0.05), showing changes over time, are for both control worm coelomic fluid and vascular blood ΣCO_2).

Table II

Effect of ethoxyzolamide on carbonic anhydrase activity in the tubeworm Riftia pachyptila

Experiment	Sample	Plume CA activity (μmol CO_2 min^{-1} g^{-1} wet wt)	Trophosome CA activity (μmol CO_2 min^{-1} g^{-1} wet wt)
Internal parameters	Control worms	103.9 ± 38.6 (5)	57.6 ± 17.9 (5)
	EZ-exposed worms	0.7 ± 0.2 (5)	0.04 ± 0.11 (5)
Flux	Control worms	82.8 ± 6.7 (2)	50.9 ± 2.1 (2)
	EZ-exposed worms	0.1 ± 0.2 (3)	0.2 ± 0.2 (3)

Carbonic anhydrase activity (mean ± SE; *n* in parentheses) for *Riftia pachyptila* in Internal *vs.* External parameter experiments and Flux experiments, with and without *in vivo* exposure to ethoxyzolamide (EZ), a carbonic anhydrase inhibitor (EZ = 0.8–0.9 mmol l^{-1} in Internal *vs.* External parameter experiments and 2 mmol l^{-1} in Flux experiments). CA activity was measured using the protocol described in Weis *et al.* (1989).

tially, the worms exhibited a net production of inorganic carbon for about 3 h (8.6 ± 0.4 μmol CO_2 g^{-1} h^{-1}; Table III, Fig. 2a). Shortly thereafter, the worms showed signs of autotrophy, indicated by net acquisition of inorganic carbon, which, again, is expressed as a negative flux rate (−12.9 ± 0.5 μmol CO_2 g^{-1} h^{-1}; Table III, Fig. 2a). During net C_i acquisition, both oxygen and sulfide uptake rates increased slightly—by 5.7% and 6.8%, respectively (Table III, Fig. 2b).

Exposure to EZ resulted in a rapid change from C_i acquisition to production (from −12.9 ± 0.5 to 8.2 ± 0.5 μmol CO_2 g^{-1} h^{-1}; Fig. 2a). The resulting net inorganic carbon production rate was similar to that seen at the beginning of the experiment ($P = 0.40$, Mann-Whitney U-test; Fig. 2a). EZ exposure also resulted in about a 99% decrease in CA activity: from 82.8 ± 6.7 to 0.1 ± 0.2 μmol CO_2 min^{-1} g^{-1} wet wt in the plume tissue and 50.9 ± 2.1 to 0.2 ± 0.2 μmol CO_2 min^{-1} g^{-1} wet wt in the trophosome (Table II). Following the addition of EZ, the worms continued to produce C_i for 3 h. During this time, however, there was a significant trend towards the re-establishment of C_i acquisition ($P < 0.0001$, Kendall rank, Fig. 2a). At the observed rate of increase, it was calculated that C_i acquisition could have been resumed within 6 h, assuming that CA inhibition was irreversible and that the return to acquisition was *via* passive diffusion.

Exposure to EZ also resulted in a transient decrease (*ca.* 50 min) in the rates of O_2 and H_2S uptake (from −16.0 to −12.2 μmol g^{-1} h^{-1} and −8.8 to −5.0 μmol g^{-1} h^{-1}, respectively; Fig. 2b). This decrease coincided with the worms retracting into their tubes, a short-term response in which the plume (the primary respiratory surface) is withdrawn from direct contact with the surrounding seawater. This often occurs when conditions are rapidly changed, and it results in a short-term (less than 60 min) decrease in flux rates. Both oxygen and sulfide flux rates returned to at least 95% of their previous values after 50 min ($P = 0.54$ and $P = 0.58$, respectively, Mann-Whitney *U* test).

Discussion

Previous studies have indicated the involvement of CA in the *R. pachyptila* symbiosis. These studies have included the measurement of CA activities in various tissues as well as the characterization of the enzyme (or enzymes), including size, kinetics, and subunit composition (Kochevar *et al.*, 1993; Kochevar and Childress, 1996). In this study, we have examined not only the levels of CA activity in these animals but also the specific functioning of this enzyme in this symbiotic association as a whole.

Carbonic anhydrase inhibition—ethoxyzolamide

In a past attempt to inhibit CA in *R. pachyptila*, Goffredi *et al.* (1997) demonstrated that exposure to sulfanilamide, another specific inhibitor of carbonic anhydrase, resulted in no significant change in coelomic fluid ΣCO_2, pH, or P_{CO_2} values. In addition, plume and trophosome samples taken from worms exposed to sulfanilamide showed no significant difference in CA activity from control worms. This is not surprising considering that sulfanilamide is a very weak, membrane-impermeable inhibitor of carbonic anhydrase, with a weak affinity for the enzyme (Maren, 1967).

Ethoxyzolamide, on the other hand, has a high affinity for carbonic anhydrase (Holder and Hayes, 1965; Maren, 1967, 1977). Exposure to EZ in our experiments resulted in a complete inhibition of CA in the trophosome and plume, a

Table III

Inorganic carbon, oxygen, and sulfide fluxes in Riftia pachyptila: *pre- and post-ethoxyzolamide (EZ) treatment*

Metabolite	Pre-autotrophic flux (μmol g^{-1} h^{-1})	Autotrophic flux (μmol g^{-1} h^{-1})	Post EZ treatment flux (μmol g^{-1} h^{-1})
Inorganic carbon	8.6 ± 0.4*	−12.9 ± 0.5	8.2 ± 0.5*
Sulfide	−8.9 ± 0.3	−9.6 ± 0.2	−9.0 ± 0.3
Oxygen	−15.9 ± 0.3	−16.9 ± 0.4	−16.0 ± 0.3

Inorganic carbon, oxygen, and sulfide fluxes (μmol g^{-1} h^{-1}) in *Riftia pachyptila* (mean ± SE, taken from Figure 2), preceding autotrophy (average over 191 min), during autotrophy (147 min), and after (post) treatment with the carbonic anhydrase inhibitor ethoxyzolamide (EZ; 205 min). The word "autotrophy" is used strictly with respect to carbon and represents a net uptake of C_i. A negative flux denotes net acquisition; a positive flux denotes net production. Asterisks denote mean fluxes of inorganic carbon that are not significantly different from one another (Mann-Whitney *U* test).

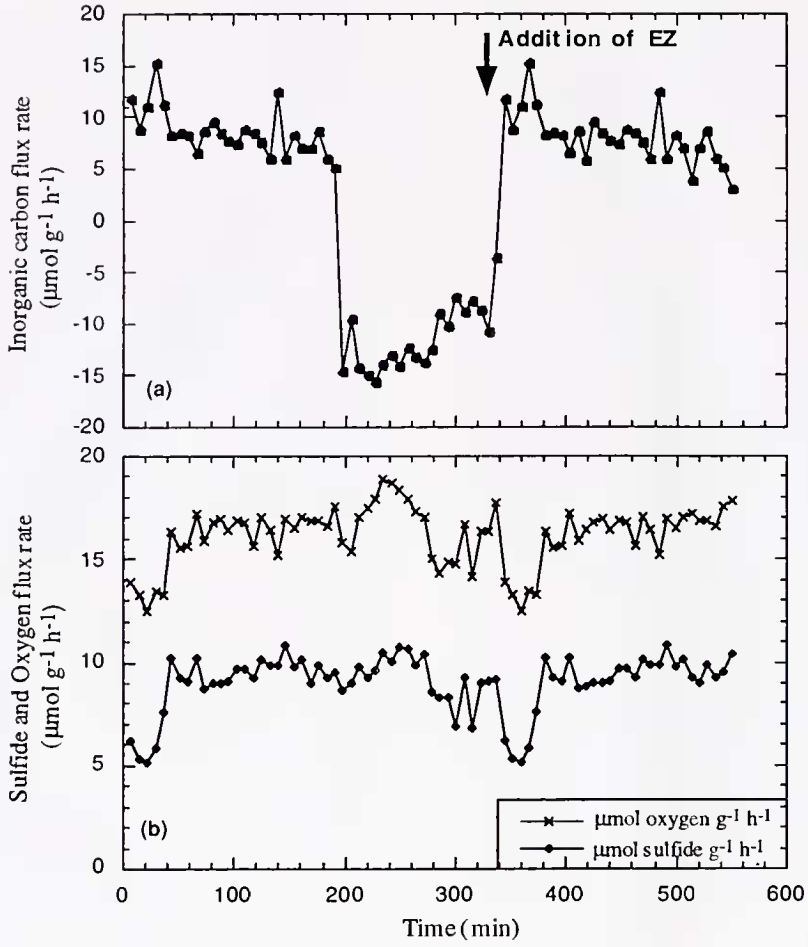

Figure 2. Plot of inorganic carbon (a) and sulfide and oxygen flux rates (b) in *Riftia pachyptila*. Each point represents the values measured at the time indicated (the entire trace represents results from one animal). Water conditions of 3.11 ± 0.32 mmol l^{-1} ΣCO_2, 0.37 ± 0.03 mmol l^{-1} ΣH_2S, 0.40 ± 0.03 mmol l^{-1} O_2, and pH of 6.25 ± 0.13 were maintained. In plot 2a, *acquisition* refers to a net uptake of inorganic carbon, and *production* refers to a net elimination of inorganic carbon. When the worm demonstrated net acquisition of inorganic carbon (*autotrophy*), ethoxyzolamide, an inhibitor of carbonic anhydrase, was added to the aquaria (2 mmol l^{-1}, arrow).

decreased rate of coelomic fluid ΣCO_2 uptake, and a complete shutdown in the movement of ΣCO_2 into vascular blood. The inhibition of CA by EZ, and the subsequent effect on carbon acquisition rates, demonstrates the importance of CA in maintaining an efficient net influx of inorganic carbon. It is interesting that the inhibition of CA by EZ resulted in net rates of CO_2 production that are similar to the pre-autotrophic rates of CO_2 production. In these experiments, freshly captured worms typically do not exhibit net inorganic carbon acquisition for the first 20 h. Thus, the net production of CO_2 during the first few hours represents CO_2 production from both host and symbiont respiration. CO_2 resulting from the metabolic processes within the association is, most likely, insufficient to meet the carbon need of the bacteria. Therefore, the acquisition of C_i from the environment is employed to help fulfill this demand, and we see this as a rather large net uptake of CO_2. Inhibition of CA results in similar rates of CO_2 production, indicating a nearly complete disruption of the carbon-concentrating system.

Until recently, it was thought that EZ was a specific inhibitor of CA, with no known properties other than CA inhibition. EZ, however, has also been suggested to inhibit proton pumps as well as CA (Maren, 1967; Harvey and Ehrenfeld, 1988). It has been demonstrated that these animals may use proton pumps to maintain an extracellular pH that is elevated in relation to the environment (Goffredi *et al.*, 1997). This creates a gradient for the inward movement of CO_2 because CO_2 demonstrates a strong pH-dependent dissociation with a pK of 6.1 (*i.e.*, the pH value at which the ratio of CO_2:HCO_3^- is 1:1; Raven, 1984; Dickson and Millero, 1987). For this reason, CO_2, the dominant chemical species *in situ* due to the acidic external pH (5–6), diffuses into the animal, which maintains an extracellular pH of

7.1–7.5 (Childress *et al.*, 1984, 1991; Goffredi *et al.*, 1997). For *R. pachyptila*, a change in the pH gradient between the internal and external environments, *via* the inhibition of proton pumps, could drastically impair ability to take up CO_2. EZ exposure in our experiments, however, did not result in significant changes in extracellular pH, indicating that EZ was not impairing extracellular pH control by inhibition of proton elimination. In previous studies, proton-pump inhibitors eliminated this pH regulation and resulted in very significant acidification of extracellular fluids with a corresponding reduction in ΣCO_2 concentrations at a given PCO_2. The fact that the EZ-inhibited worms showed no reduction in extracellular fluid pH, under conditions in which worms treated with other proton pump inhibitors showed very significant decreases, strongly suggests that EZ did not inhibit the relevant proton ATPases (Goffredi *et al.*, 1999). Thus, after a period of exposure to elevated P_{CO_2}, the lower extracellular ΣCO_2 and lower rates of C_i accumulation in the extracellular fluids are almost certainly due to a lower rate of movement of C_i into the extracellular fluid. Inhibition of CA is the most likely cause of this reduced rate of movement; therefore based on these results, we believe that EZ acted primarily upon carbonic anhydrase.

In our flux experiments, the addition of EZ resulted in an initial decrease in net acquisition of C_i. After the onset of inhibition, however, there was a significant trend towards restoration of net C_i acquisition over time. The initial decrease in net C_i acquisition was presumably caused by the permanent inhibition of CA by EZ; however, this inhibition did not significantly affect the extracellular pH. Under normal conditions (*i.e.*, without an inhibitor), CA is apparently essential for facilitating C_i uptake at the CO_2 gradient that exists when the worm exhibits autotrophy. However, with the complete inhibition of CA, this gradient is no longer sufficient for the net uptake of CO_2. We would expect, therefore, that as the EZ-exposed *R. pachyptila* depletes the vascular and coelomic C_i pools, the increasing CO_2 gradient would lead to an eventual reduction of net C_i production, as seen in our experiments. About 20% of the total C_i acquisition (heterotrophic C_i production − autotrophic C_i uptake) by *R. pachyptila* had been recovered at 4 h following the addition of EZ. To determine the rates that would have been achieved without the functioning of CA, longer experiments would have been necessary.

Carbon-concentrating mechanisms

Judging from the results of our animal metabolism experiments, it appears that CA plays an important role in the CO_2-concentrating mechanism in *R. pachyptila*. Prior studies have addressed the importance of proton elimination in maintaining a diffusion gradient for the influx of CO_2 (Goffredi *et al.*, 1997). The elevated extracellular pH maintained by these worms allows for C_i to be concentrated in the extracellular fluid above external levels, *via* two mechanisms. The process of pumping protons into the surrounding medium, which has been demonstrated in *R. pachyptila*, increases the free CO_2 concentration externally, which creates a larger external pool of CO_2 available to diffuse inward (Raven, 1991; Girguis and Childress, 1996). Secondly, the actual pH gradient maintained between the internal (pH 7.3) and external environments (pH 6.0) acts to concentrate C_i internally by shifting the internal C_i equilibrium towards HCO_3^-. Under steady-state conditions, the interconversion of CO_2 to bicarbonate at the plume must be reversed in the trophosome because CO_2 is the species of C_i utilized by the symbionts of *R. pachyptila* (Scott *et al.*, 1994). Thus, the gradient for diffusion must ultimately be created by C_i consumption by the symbionts. This provides the "driving force" for the passive influx of CO_2 but does not expedite the conversion of CO_2 into bicarbonate.

By speeding the interconversion between CO_2 and HCO_3^-, CA helps to maximize the effectiveness of CO_2 movement into the plume, through the animal, to the symbionts, allowing for very effective acquisition of inorganic carbon from the environment. Although CO_2 transport at the water/epithelium interface is accomplished by diffusion of dissolved CO_2, an important role for CA in the equilibration between C_i in the external and internal environments is likely, given its high concentrations and the high rates of inorganic carbon uptake. It appears that both the maintenance of a strong pH gradient and high levels of carbonic anhydrase work in concert to rapidly concentrate C_i in this animal, thus providing the symbionts with an abundance of CO_2. Ability to maintain these conditions is apparently a further specialization of *Riftia pachyptila* for supporting autotrophic endosymbionts.

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Literature Cited

Arp, A. J., J. J. Childress, and C. R. Fisher, Jr. 1985. Blood gas transport in *Riftia pachyptila*. *Bull. Biol. Soc. Wash.* **6:** 289–300.

Arp, A. J., J. J. Childress, and R. D. Vetter. 1987. The sulphide-binding protein in the blood of the vestimentiferan tube-worm, *Riftia pachyptila*, is the extracellular haemoglobin. *J. Exp. Biol.* **128:** 139–158.

Badger, M. R. 1987. The CO_2-concentrating mechanism in aquatic phototrophs. Pp. 219–274 in *The Biochemistry of Plants: A Comprehensive Treatise,* Vol. 10, M. D. Hatch and N. K. Broadman, eds. Academic Press, New York.

Burnett, L. E. 1984. CO_2 excretion across isolated perfused crab gills: facilitation by carbonic anhydrase. *Am. Zool.* **24:** 253–264.

Burnett, L. E., and B. R. McMahon. 1985. Facilitation of CO_2 excretion by carbonic anhydrase located on the surface of the basal membrane of crab gill epithelium. *Respir. Physiol.* **62:** 341–348.

Childress, J. J., A. J. Arp, and C. R. Fisher, Jr. 1984. Metabolic and blood characteristics of the hydrothermal vent tube worm *Riftia pachyptila. Mar. Biol.* **83:** 109–124.

Childress, J. J., C. R. Fisher, J. A. Favuzzi, R. E. Kochevar, N. K. Sanders, and A. M. Alayse. 1991. Sulfide-driven autotrophic balance in the bacterial symbiont-containing hydrothermal vent tubeworm, *Riftia pachyptila* Jones. *Biol. Bull.* **180:** 135–153.

Childress, J. J., R. Lee, N. K. Sanders, H. Felbeck, D. Oros, A. Toulmond, D. Desbruyéres, M. C. Kennicutt II, and J. Brooks. 1993. Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental pCO_2. *Nature* **362:** 147–149.

Dickson, A. G., and F. J. Millero. 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in sea water media. *Deep-Sea Res.* **34:** 1733–1743.

Girguis, P. R., and J. J. Childress. 1996. Proton equivalent ion flux in *R. pachyptila.* (Annual Meeting of the Society for Integrative and Comparative Biologists, Albuquerque, New Mexico, USA, December 26–30, 1996). *Am. Zool.* **36(5):** 64A.

Goffredi, S. K., J. J. Childress, N. T. Desaulniers, R. W. Lee, F. H. Lallier, and D. Hammond. 1997. Inorganic carbon acquisition by the hydrothermal vent tubeworm *Riftia pachyptila* depends upon high external P_{CO_2} and upon proton elimination by the worm. *J. Exp. Biol.* **200:** 883–896.

Goffredi, S. K., J. J. Childress, F. H. Lallier, and N. T. Desaulniers. 1999. The internal ion composition of the hydrothermal vent tubeworm *Riftia pachyptila:* evidence for the elimination of SO_4^{2-} and H^+ and for a Cl^-/HCO_3^- shift. *Phys. Zool.* **72** (in press).

Harvey, B. J., and J. Ehrenfeld. 1988. Epithelial pH and ion transport regulation by proton pumps and exchangers. Pp. 139–164 in *Proton Passage Across Cell Membranes.* G. Bock and J. Marsh, eds. John Wiley, Chichester, UK.

Henry, R. P. 1987. Membrane-associated carbonic anhydrase in gills of the blue crab, *Callinectes sapidus. Am. J. Physiol.* **252:** R966–R971.

Henry, R. P. 1988. Multiple functions of carbonic anhydrase in the crustacean gill. *J. Exp. Zool.* **248:** 19–24.

Holder, L. B., and S. L. Hayes. 1965. Diffusion of sulfonamides in aqueous buffers and into red cells. *Mol. Pharmacol.* **1:** 266–279.

Kochevar, R. E. 1992. Carbonic anhydrase in the giant hydrothermal vent tubeworm *Riftia pachyptila* Jones and other deep-sea chemoautotrophic symbioses. Ph.D. dissertation, University of California, Santa Barbara. 103 pp.

Kochevar, R. E., and J. J. Childress. 1996. Carbonic anhydrase in deep-sea chemoautotrophic symbioses. *Mar. Biol.* **125:** 375–383.

Kochevar, R. E., J. J. Childress, C. R. Fisher, and L. Minnich. 1992. The methane mussel: roles of symbiont and host in the metabolic utilization of methane. *Mar. Biol.* **112:** 389–401.

Kochevar, R. E., N. S. Govind, and J. J. Childress. 1993. Identification and characterization of two carbonic anhydrases from the hydrothermal vent tube-worm *Riftia pachyptila* Jones. *Mol. Mar. Biol. Biotechnol.* **2:** 10–19.

Lowry, O. H., N. J. Rosebrough, L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **193:** 267–275.

Lucas, W. J., and J. A. Berry. 1985. Inorganic carbon transport in aquatic photosynthetic organisms. *Physiol. Plant.* **65:** 539–543.

Lutz, R. A., T. M. Shank, D. J. Fornari, R. M. Haymon, M. D. Lilley, K. L. Von Damm, and D. Desbruyéres. 1994. Rapid growth at deep-sea vents. *Nature* **371:** 663–664.

Maren, T. H. 1967. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol. Rev.* **47:** 595–779.

Maren, T. H. 1977. Use of inhibitors in physiological studies of carbonic anhydrase. *Am. J. Physiol.* **232:** F291–F297.

Meldrum, N. U., and F. J. W. Roughton. 1933. Carbonic anhydrase: Its preparation and properties. *J. Physiol.* **80:** 113–142.

Raven, J. A. 1984. *Energetics and Transport in Aquatic Plants.* A. R. Liss, New York.

Raven, J. A. 1991. Implications of inorganic carbon utilization: ecology, evolution, and geochemistry. *Can. J. Bot.* **69:** 908–924.

Scott, K. M., C. R. Fisher, J. S. Vodenichar, E. R. Nix, and E. Minnich. 1994. Inorganic carbon and temperature requirements for autotrophic carbon fixation by the chemoautotrophic symbionts of the giant hydrothermal vent tube worm, *Riftia pachyptila. Physiol. Zool.* **67:** 617–638.

Toulmond, A., F. H. Lallier, J. de Frescheville, J. J. Childress, R. Lee, N. K. Sanders, and D. Desbruyéres. 1994. Unusual carbon dioxide-combining properties of body fluids in the hydrothermal vent tubeworm *Riftia pachyptila. Deep-Sea Res.* **41:** 1447–1456.

Weis, V. M. 1991. The induction of carbonic anhydrase in the symbiotic sea anemone *Aiptasia pulchella. Biol. Bull.* **180:** 496–504.

Weis, V. M., G. J. Smith, and L. Muscatine. 1989. A "CO_2 supply" mechanism in zooxanthellate cnidarians: role of carbonic anhydrase. *Mar. Biol.* **100:** 195–202.